Vomitoxin: Natural Occurrence on Cereal Grains and Significance as a Refusal and Emetic Factor to Swine

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Fifty 12,13-epoxytrichothecenes have been reported in the literature; of these diacetoxyscirpenol, T-2 toxin, nivalenol and vomitoxin have been naturally occurring in cereal grains throughout the world. Vomitoxin is produced in ears of corn prior to harvest when wet, cool weather precedes which favours *Gibberella zeae* growth. This type of infected corn, which often contains vomitoxin, is associated with the refusal and emetic syndrome exhibited by swine.

Since 1916, emesis in humans resulting from ingestion of food prepared from *Fusarium*-infected cereal grains has been recorded in several areas around the world. In the Soviet Union, it was known as the drunken bread intoxication. Sporadic food poisoning featuring vomiting, nausea, somnolence, headache and convulsion occurred in Japan during the 1950's and the late 1940's. These disorders were associated with consumption of rice contaminated with *Gibberella zeae* and *Fusarium nivale*[1] and of foods made from *G. zeae* infected wheat flour[2]. In the southern part of Korea in 1963, barley infected with *F. graminearum* was reported to cause in humans the toxic disturbances of nausea, vomiting, abdominal pains and diarrhoea[3]. Comparable symptoms were observed in swine and a few cattle. In a similar phenomenon, involving swine consuming corn naturally contaminated with *F. graminearum* in the U.S., a new trichothecene, vomitoxin, was isolated and shown to be the causative agent[4]. Whether or not the factor causing emesis in humans is identical to that in swine is unknown, particularly since several trichothecenes have been capable of causing various laboratory animals to vomit[2,5,6]. Vomitoxin was also shown to be the principal toxin responsible for the phenomenon of feed refusal exhibited by swine[7].

History of refusal and emetic factors in scabbed grains

Cereal grains that cause swine or farm animals with simple stomachs to vomit on ingestion and refuse feed usually connotes a scab condition in grain. This disease in grains is indicated by growth of the mould *G. zeae* (perfect stage of *F. graminearum*) on the kernels and flowering head. On corn, *G. zeae* occurs as a reddish mould that begins as observable growth on the ear tip. This condition is referred to as *Gibberella* or pink ear rot[8].

Outbreaks of barley scabbed with *G. zeae* in the Midwest region of the U.S. were reported in 1919, 1928[9] and in the 1930's[10]. This barley was rejected by swine. Barley from the 1928 U.S. crop exported to Germany was also toxic to swine. Swine force-fed this barley became sick and vomited, and eventually death ensued from preference not to eat. Equines also rejected feed containing mouldy barley, but cattle and chickens were not susceptible. Farm animal disorders due to ingestion of scabbed barley in the U.S. subsided as production moved from the humid Midwest environment to the West.

Wheat also is susceptible to *Gibberella* infection, but its involvement in the swine syndrome of refusal and emesis appears to be less frequent than that of barley and corn. Fusaria-infected corn now seems to be the major grain in the U.S. which has detrimental effects on swine performance. In 1972, corn contaminated with *G. zeae* caused extensive losses to swine operations, reaching epidemic proportions in the U.S. Sporadic Fusaria outbreaks in the corn belt also occurred in Indiana in 1958 and 1965[11] and in northwest Ohio in 1970, 1975 and 1977[12]. Low temperature with concomitant high moisture conditions, which usually results in a delayed harvest, appears to favour *G. zeae* corn infection. This was the situation in 1966 and 1972. In addition, the 1972 corn crop was not dried immediately because of energy shortages for operating dryers; this introduced a storage variable.

Unusually wet weather preceded harvest in northwest Ohio in 1977. Conditions were conducive to *G. zeae* growth on corn intended for swine feed, and much of this corn was found to contain vomitoxin[12]. Even with these documented cases, information concerning environmental conditions and factors governing production of refusal and emetic factors on grains infected with Fusaria in the field is meagre. The ability of *G. zeae* to render grains unwholesome for consumption by swine and other farm animals may be dependent on the type of hybrid, but studies of whether *G. zeae* infection can be controlled by breeding new plants have not been conducted.

Emetic substances were extracted from naturally moulded barley with water[3,9,13]. A water extract obtained from barley infected with *G. saubentii* has been reported to cause vomiting when intubated into swine[14]. Swine also refused feed made from barley naturally infected with Fusaria and barley cultured with *G. saubenetti.* Water extracts from *F. graminearum*-contaminated barley administered intraperitoneally (ip) caused toxic disturbances in suckling mice and pigs but had no apparent effect on rabbits, rats, mature mice and day-old chicks[3].

G. zeae-infected corn (12 to 100% of kernels were infected) produced in northern Indiana in 1965 has been investigated for emetic and refusal factors[15]; hog farmers reported that this corn was refused by swine and caused a few pigs to vomit. In feeding tests, swine rejected the naturally infected corn and the corn amended with water extracts obtained from this infected corn. In addition, swine vomited when this water extract was administered by stomach tube, intravenously or ip. The substances extracted with water from this infected corn were separated into two fractions, based on their solubility in methanol. Swine ingesting corn amended with the methanol-soluble fraction vomited initially but would return to eat without further symptoms of nausea or other effects. Hence, it was proposed that the water extract of *G. zeae*-infected corn contained a methanol-soluble emetic factor and a methanol-insoluble refusal factor.

Emetic substances associated with Fusaria species isolated from cereals have been reported, using pigeons as an assay tool[16]. Emetic factors were produced on corn inoculated in the field with *F. graminearum* and from liquid culture media fermented with a strain of *F. moniliforme, F. roseum, F. poae, F. culmorum* and *F. nivale.* The emetic principle elaborated by *F. monili-*

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forme was lethal to the pigeon in 12 hours when injected through the wing vein. In Japan, at about the same time, nivalenol, a trichothecene produced by *F. nivale* cultured on liquid media was reported[2,17]. This *Fusarium* strain was isolated from mouldy rice that caused nausea and vomiting in 25 young people. The emetic-producing Fusaria strains described subsequently received much attention by investigators throughout the world. In a screen of Fusaria species using chemical and bioassay methods, it was found that the *F. poae* strain produced the trichothecenes T-2 toxin, HT-2 toxin and neosolaniol on a peptone-supplemented Czapek-Dox media at 25 and 27°C[18]. Trichothecenes were not detected in the culture liquor for *F. moniliforme* and *F. nivale* strains. The PD strains on moist corn at 5 and 25°C and on Richards solution at 25°C have been studied[5]. Extracts obtained from the *F. poae* strain cultured on corn at 8°C and from the Richards solution induced emesis in pigeons either by intubation into the crop or intravenous injection through the wing at a non-lethal concentration. The emetic was identified as T-2 toxin. Acetyl T-2 toxin was isolated from shaken liquid cultures of *F. poae* and shown to be a less potent emetic to pigeons than T-2 toxin[19]. The role of acetyl T-2 toxin in the syndrome of emesis and refusal in swine remains obscure, since it has not been detected in feedstuffs, in cereal grains or on corn cultured with Fusaria species in the laboratory.

The conjecture was made that, since only the *F. poae* strain produced emetic factors, perhaps the other four PD strains may no longer be identical with the original strains and that T-2 toxin and acetyl T-2 toxin may be the emetics PD described. Each PD strain on autoclaved corn at 28°C was re-examined for ability to elaborate rejection and emetic factors. Each corn sample was evaluated for toxic factors by a pig bioassay based on the degree of acceptance of corn amended with extracts obtained from the cultured corn[7,21]. Corn cultured with each PD strain was refused by swine. Analyses of the refused corn for trichothecenes showed that the corn fermented with *F. culmorum* contained vomitoxin. The method of detection was a thin-layer chromatographic procedure that was rather insensitive for the trichothecenes T-2 toxin, HT-2 toxin, acetyl T-2 and fusarenone-X. These strains are being re-investigated for their ability to produce toxins by extensive isolation procedures and the detection system of gas chromatography-mass spectrometry (GC-MS).

The *Fusarium* outbreak in U.S. corn in 1972, which was associated with swine refusal to eat this contaminated corn, perplexed investigators around the world as to whether toxic factors were involved. Contaminated predominantly with *F. graminearum,* corn produced in northwest Ohio, which caused swine to vomit on ingestion followed by refusal to eat was shown to contain a new trichothecene, vomitoxin[4]. *F. graminearum* isolates from this corn elaborated vomitoxin on autoclaved grain, and vomitoxin was demonstrated as the major emetic and refusal factor to swine[7]. A level of 40 ppm of vomitoxin was found in the northwest Ohio field corn. The natural occurrence of vomitoxin was subsequently reported in the same area in 1977[22]. In four of nine corn samples collected by the Food and Drug Administration from the Midwest in 1972, vomitoxin was found at levels ranging from 15-28 ppm. These findings show vomitoxin to be the main trichothecene involved in the syndrome of refusal and emesis exhibited by swine in the U.S. in 1972.

Classification of Fusaria involved in fusariotoxicoses

Since Link's description of the genus *Fusarium* in 1809, classification of this species has followed many schemes: The thousand Fusaria that had been described by the 1930's was lumped into sixteen groups, six subgroups and 142 species varieties[23]; About 50 species were recognised in the *Fusarium* genus[24]; All Fusaria were reduced to nine species[25,26]; and there are several other classification systems[27,28]. The differences in opinion among mycologists in classifying Fusaria species necessitate identification of the system followed to describe this species. In the mycotoxicological literature synonomous names of Fusaria, dependent upon classification system, are: *F. roseum* = *F. graminearum* = *F. equiseti* = *F. scirpi* = *F. avenaceum* = *F. culmorum* (Section: Roseum, Gibbosum, Fusarium, Discolor); *F. tricinctum* = *F. poae* = *F. sporotrichioides* = *F. sporotrichiella* (Section: Sporotrichiella).

Fusarium species that have the ability to parasitize plants are indigenous in soil fungi. Fusaria of the Sporotrichiella section and Roseum section have been isolated from cereal grains; toxins produced by these species, which have been associated with the disorders in farm animals, are: T-2 toxin causes a hemorrhagic syndrome in swine and cattle[29]; zearalenone causes a hyperestrogenism condition in swine[30]; vomitoxin is a potent refusal and emetic in swine[7]; leukoencephalmalacia is associated with equine ingestion of corn contaminated with *F. moniliforme* (Section: Liseola), and the factor is unidentified[31]. Fusaria of the Sporotrichiella section are found less frequently on corn at harvest in temperate zones, but it is more of a problem on cob corn stored in a crib and overwintered. However, colonists of the Roseum, Gibbosium or Fusarium (Discolor) section are common on corn in the U.S.

Physiochemical properties of vomitoxin

Vomitoxin characterized as 3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one[4,7] is also referred to as R_d toxin and 4-deoxynivalenol[32]. The latter trivial name implies that the vomitoxin structure is like nivalenol but lacks a C_4 hydroxy group. Vomitoxin ($C_{15}H_{20}O_6$), a white solid crystallized from ethyl acetate and hexane as fine needles mp 153-154°C; $(\alpha)_D^{26}$ + 6.5 (C 0.07 ethanol), is soluble in ethyl acetate, methanol, ethanol and chloroform; insoluble in hexane; and slightly soluble in water. The ultraviolet absorption of vomitoxin in methanol showed a maxima at 226, and its infrared spectrum showed absorption at 3340-3460 broad, 2950 and 1680 cm^{-1}.

The nuclear magnetic resonance spectrum of vomitoxin in $CDCl_3$ (tetramethylsilane as internal standard) gave the following chemical shifts: δ 1;13 s, 3 H, $\underline{CH_3}$-C; 1.88 bs, 3H, $\underline{CH_3}$-C=C; 2.16 m, 2H, $\underline{CH_2}$; 3.10 ABq, J=4 Hz, $\underline{CH_2}$; 3.60 d,1H, $\underline{CH}$; 3.82 s, 2H, $\underline{CH_2}$-OH; 4.50 m, 1H, $\underline{CH}$-OH; 4.80 m, 1H, $\underline{CH}$-OH; O-$\underline{CH}$-HC=C; 6.60 m, $\underline{CH}$=C. The mass spectrum of vomitoxin showed a molecular ion at 296.157, and loss of CH_3, H_2O, hydroxymethyl or formaldehyde as characterized by fragment ion peaks at m/e 281, 278, 265 and 286, respectively. The most prominent ion, at m/e 248, which corresponds to loss of both water and formaldehyde, is consistent for keto at C-8, hydroxyl at C-7 and hydroxymethyl at C-6.

Vomitoxin was converted into its triacetate with acetic anhydridepyridine (m.p. 155-156°C) with a molecular ion 422.155. The NMR spectrum in $CDCl_3$: δ 0.96 s, $\underline{CH_3}$; 1.86 s, 3H, $\underline{CH_3}$; 1.90 s, 3H, $\underline{CH_3}$; 2.12 s, 3H, $\underline{CH_3}$; 2.20 s, 3H, $\underline{CH_3}$; 2.95 ABq, J=4, Hz 2H, $\underline{CH_2}$-O; 3.89 d, $\underline{CH_2}$, 5.2 m, 1H, $\underline{CH}$-OAc; 4.30 s, 2H, $\underline{CH_2}$OAc; 4.76 d, J=5.5, $\underline{CH}$-CH=C; 6.50 d, J=5.5, $\underline{CH}$=C; 6.04 s, 1H, $\underline{CH}$-OAc.

Natural occurrence of vomitoxin and trichothecenes

Prior to the finding of vomitoxin occurring naturally in corn and its documentation as the agent responsible for emesis and refusal syndrome exhibited by swine[4,7], 2 ppm of T-2 toxin was reported in feed associated with a lethal toxicoses in dairy cattle[29]. Subsequently, these two trichothecenes, as well as diacetoxyscirpenol (DAS) and nivalenol (NIV), have been found in cereals throughout the world. Table 1 summarizes the natural occurrence of vomitoxin, and Table 2 cites incidence of T-2 toxin, DAS and NIV. In a survey of corn from the 1972 crop for *Fusarium* toxins, it was found that extracts from 93 samples caused a dermal reaction by the rabbit skin bioassay[42]. The dermatitic effect was attributed to T-2 toxin, although it was not detected. Nine of these samples were re-examined, six of which produced a skin irritation to the rabbit[22]. Vomitoxin levels of 15 to 28 ppm were found in four samples, but T-2 toxin and DAS were not detected in the nine samples by GC-MS. Vomitoxin was detected in Austrian and Canadian corn refused by swine[33] and also has been reported in corn in France[37] and South Africa[38] and in barley in Japan[42].

Vomitoxin levels ranging from 0.40 to 1.8 ppm were reported in feedstuffs refused by swine and T-2 toxin at a lower level, as well as DAS and zearalenone[36]. Since zearalenone often occurs in corn naturally contaminated with *G. zeae,* it has been suggested that zearalenone may enhance the refusal syndrome[38]. In a mouse bioassay, based on their being able to reject drinking water containing trichothecenes, zearalenone (100 mg/l) did not enhance the refusal response for low levels (2 mg/l) of either T-2 toxin, DAS or vomitoxin[48].

Analysis of vomitoxin

Trichothecenes such as vomitoxin and nivalenol, which contain three and four hydroxyl groups, respectively, and a carbonyl at the C_8 position, have been extracted from grains with combinations of polar solvents. Vomitoxin was separated

Table 1. Natural occurrence of vomitoxin on cereal grains and feed

Country	Commodity	Concentration μg/g	Reference
USA	Corn	40	7, 22
USA	Corn	15, 18, 20, 28	22
USA	Corn	28	33
USA	Corn	0.7	34
USA	Corn	12	35
USA	Corn	a	12
USA	Corn	1, 1.8	36
USA	Commercial, mixed feed	0.04-0.06	36
USA	Mixed feed	0.4, 0.6, 1	36
Japan	Barley	7.3	32
France	Corn	0.1-0.6	37
Canada	Corn	7.9	33
Canada	Feed	1.2	33
Austria	Corn	1.3, 7.9	33
Austria	Feed	+	33
Austria	Corn	b	47
South Africa	Corn	2.5	38
Zambia	Corn	7.4	38

[a] Vomitoxin was detected in a range of 0.5 to 10 μg/g in 24 of 52 corn samples from Northwest Ohio.
[b] Vomitoxin found in range of 1 to 20 μg/g in 56 maize samples obtained from Styria, Austria.
+ = Not quantified.

Table 2. Natural occurrence of T-2 toxin, nivalenol (NIV) and diacetoxyscirpenol (DAS) on cereal grains

Country	Commodity	Concentration μg/g	Reference
USA	Corn	T-2:2	29
USA	Mixed feed	T-2:0.076, DAS:0.5	36
Canada	Barley	T-2:25	39
France	Corn	T-2:0.02, NIV:4.8	37
Germany	Corn	DAS:31.5	40
India	Sweet corn	T-2:4, DAS:14	41
Japan	Barley	NIV:detected	32

from corn with 40% aqueous methanol[4] and vomitoxin and nivalenol from barley with 50% aqueous ethanol[32]. Quantitation of trichothecenes by gas-liquid chromatography (GLC) and GC-MS used in the selected ion mode is most attractive due to increased sensitivity, precision and reliability.

The authors have used a quantitative GLC method for vomitoxin with internal standard[12]. The following procedure was used to obtain sample extract: The corn sample (50-300 g) was dried 4 hr at 78°C. Each dried sample was blended with butanol in a blender (1 g/2 ml). This extraction step removes corn oil, lipids and pigments in addition to other butanol soluble substances. The corn freed of butanol solubles was blended with 40% aqueous methanol (1 g/2 ml). Each sample was extracted twice by this procedure. The combined CH_3-OH:H_2O extracts was evaporated to dryness, and the remaining residue was chromatographed on a silica gel column (10-30 g) presaturated with $CHCl_3$. The column was eluted consecutively with $CHCl_3$, $CHCl_3$:CH_3OH (90:10). The latter column eluate was analyzed for vomitoxin by GLC on a column packed with 3% OV-101 on Gas Chrom after addition of trimethyl silating (TMS) reagent and the internal standard DAS. Column temperature was programmed from 160-250°C at 5°C/minute. Vomitoxin retention time relative to the TMS derivative of DAS was 1.3 minutes. A weight ratio range of vomitoxin/DAS of 0.5/1 to 1.5/1 was used. Quantitation of the TMS derivative of vomitoxin by GC:MS by the calibration method in the SIM mode has been reported[22]. Using a computerized GC:MS in the SIM mode vomitoxin, DAS, T-2 toxin, and zearalenone in feedstuffs was found[36]. Ethyl acetate extracted T-2 toxin, DAS and zearalenone, and the polar solvent CH_3OH:H_2O extracted vomitoxin from feedstuffs.

Visualization of trichothecenes on TLC plates has been made after spraying with p-anisaldehyde reagent[43]; colour changes of trichothecenes with a carbonyl at the C_8 position were described as nonfluorescent brown spots after spraying with H_2SO_4.[44] These spray reagents may be useful during the isolation of a particular trichothecene and may provide information on the degree of purity. Vomitoxin turns yellow to p-anisaldehyde spray reagent after heating at 110° for a few minutes, and with H_2SO_4 it turns a yellow brown colour. The Rf values of vomitoxin are 0.7 in $CHCl_3$:CH_3OH (8.2 v/v) and 0.1 in toluene: ethyl acetate:90% formic acid (6:3:1 v/v/v).

Recently, it has been reported that 4-p-nitrobenzyl-pyridine[45] produces a blue spot on a white background on TLC plates for a number of trichothecenes (vomitoxin 0.1 μg, nivalenol 0.05 μg, DAS 0.2 μg and T-2 toxin 0.1 μg). DAS turns blue purple with p-dimethylaminobenzaldehyde[46] in hydrochloric acid in ethanol.

The skin reaction response of small laboratory animals in response to trichothecenes is appealing as a bioassay because of its simplicity, reliability and sensitivity. Vomitoxin applied in ethylacetate to shaved backs of rabbits produced a visible vesication at 1 μg, but no irritation appeared on the shaved backs of mice (Vesonder, unpublished results). The animal skin test may be expedient to eliminate from further study samples that test nevative; however, a positive test can result from compounds other than trichothecenes.

Vomitoxin - - Economics

The methodology for extraction of trichothecenes such as vomitoxin and nivalenol from cereal grains is complex, tedious, laborious and time consuming. The present state of the art for isolation of trichothecenes from grains poses many questions concerning quantitative determinations. Further, this class of compounds has exacerbated precise chemical analytical methods due to their nonfluorescent properties and their lack of intensity in the ultraviolet and infrared region of the spectrum. Hence, quantitation measurements have been limited to sophisticated equipment (e.g., GC:MS and GLC).

Nevertheless, even though individual assessment of vomitoxin occurrence in cereal grains is meagre, circumstantial evidence indicates that economic losses have occurred to swine farmers because of this toxin. Farmers in northwest Ohio in 1975 reported a major portion of their corn was refused by swine; this corn was heavily infected with *G. zeae* and had to be replaced with corn the swine would eat. The corn supply became short from grain elevators in this area and had to be obtained from suppliers as far as Peoria, Illinois, at an additional cost of $0.50/bushel. The losses sustained by the swine farmers in northwest Ohio were staggering, and many faced financial disaster. As a result of this severe *G. zeae* outbreak in 1975, northwest Ohio farmers now inspect ears of corn in the field for visible signs of this mould. If the corn appears to have this type of mould infection, it is withheld from feeding to swine, but can be a part of the feed given to cattle or chickens which are not affected by this type corn. In 1977, when preharvest corn in a four-county area in northwest Ohio was surveyed, the farmers reported that much of it was contaminated with *G. zeae*. Infected kernels ranged from 2 to 50% for 44 samples, and eight showed none. An incidence of 46% vomitoxin contamination was found in these samples collected from 26 farms in northwest Ohio. This survey established that vomitoxin is produced in the field when wet and cool weather persists before harvest. Even higher levels of vomitoxin might have occurred if a rainy period had delayed harvest. Such a situation did occur in 1972 in the corn belt of the United States, with a high level of *Fusarium* infection. Since corn rejected by swine is often fed to animals that can tolerate it, the actual economic losses from vomitoxin in swine rations or the presence of other trichothecenes may not be accurately estimated.

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